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RAPID METHOD FOR THE DETERMINATION AND QUANTIFICATION OF BROMOSULPHOPHTHALEIN AND METABOLITES IN CULTURED HEPATOCYTES, CULTURE MEDIA AND BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

G.A.E. VAN 't KLOOSTER, J.H. BOOT, W.C. MENNES and B.J. BLAAUBOER*

Institute of Veterinary Pharmacology, Pharmacy and Toxicology, University of Utrecht, P.O. Box 80176, 3508 TD Utrecht (The Netherlands)

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SUMMARY

An ion-pair high-performance liquid chromatographic method for the rapid, selective and sensitive analysis of samples containing bromosulphophthalein (BSP) and its conjugates is presented. The method is useful for analysis in bile, culture media and cultured hepatocytes. Two sample preparation methods are described. Even though BSP recovery from albumin binding is complete, only a small percentage of free BSP can be detected in cells, possibly owing to a conjugation-related pool of BSP in cells. As BSP-glutathione recovery is complete, the method offers a useful tool to investigate impairment of glutathione conjugation.

INTRODUCTION

The organic dye bromosulphophthalein (BSP, Fig. 1) is a well known compound frequently used in clinical tests of liver functioning and as a model reagent

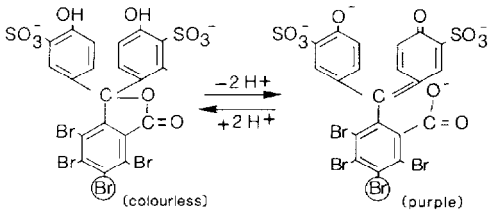

Fig. 1. BSP structure and chromophore formation. In alkaline solutions BSP is deprotonated with opening of the lactone ring ($pK_a = 8.8$). The resulting compound strongly absorbs visible light at 578 nm. The encircled bromine atom is replaced by glutathione on conjugation; the chromophore is not affected.

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to investigate transport and metabolism in liver cells of compounds execreted in the bile [1–3]. BSP is excreted via the bile, a process that involves uptake in parenchymal liver cells, intracellular binding, formation of BSP–glutathione conjugates (BSP-S-G) mediated by glutathione-S-transferases, and finally secretion into the bile [1–8].

Isolated hepatocytes both in suspension and in primary culture offer a very useful system in toxicological studies [9]. The effects of compounds interfering with glutathione conjugation on the transport and metabolism of BSP may be investigated in such a system. This demands a method of determination and quantification of BSP as well as its conjugates.

In alkaline solution BSP ($pK_a = 8.8$) can easily be determined and quantified in a specific and sensitive way by spectrophotometry, owing to its large specific extinction at the absorption maximum (578 nm). All clinical methods proposed for determination of the dye take advantage of this fact [10]. Unfortunately, both the absorption spectra between 500 and 650 nm and the extinction coefficients of BSP and its conjugates are identical [1,11], which thwarts an easy simultaneous spectrophotometric quantification of both compounds.

Spectrophotometric measurement preceded by separation methods by means of paper chromatography [1,5,12] and thin-layer chromatography (TLC) [2,3,8,13] have been reported. Experiments in the lower concentration ranges were performed using ^{35}S-labelled BSP in combination with TLC [2,3,6,8]. These methods have their limitations, such as lack of sensitivity or accuracy, lack of general applicability and a need for special laboratory facilities.

Chen et al. [3] described a high-performance liquid chromatographic (HPLC) method for separation and quantification in bile using spectrophotometric detection at 254 nm. They reported their method to be useful neither in culture media nor in intracellular fluid, especially in the lower concentration range.

Even at pH 2, the lowest allowable pH for column packing materials, BSP and its conjugates are doubly ionized because of the sulphonic acid groups (Fig. 1). The employment of ion-pairing reagents to bring about retention of sulphonated dyes on common reversed-phase columns has been described by Gloor and Johnson [14].

We evolved a rapid ion-pair HPLC method for the separation and quantification of BSP and its conjugates. Sensitive and specific analysis of these compounds was achieved by post-column alkalinization of the effluent and spectrophotometric detection at 578 nm.

EXPERIMENTAL

Materials

BSP was purchased from E. Merck (Darmstadt, F.R.G.). Reduced glutathione (GSH) was obtained from Sigma (St. Louis, MO, U.S.A.).

BSP-S-G was synthesized according to Whelan et al. [1] as well as isolated from bile produced by in situ perfused rat liver. In both cases products were purified by means of TLC (Merck silica gel F254, 0.25 mm thickness; 2-methyl-2-propanol–distilled water (3:1) as proposed by Sardini et al. [13]).

Tetrabutylammonium hydroxide (0.4 *M* in water, HPLC grade) and tetraethylammonium hydroxide were from Eastman Kodak (Rochester, NY, U.S.A.). Tetramethylammonium chloride was purchased from Fluka (Buchs, Switzerland).

All other chemicals were of analytical grade or better.

Animal treatment and sample preparation

Rat (male Wistar; 225 g) hepatocytes were isolated using the two-step perfusion technique described by Seglen [15] as modified by Paine et al. [16]. Bile was collected from rat liver, perfused in situ for 1.5 h with 40 μM BSP in Hanks' balanced salt solution (HBSS), saturated with oxygen–carbon dioxide (95:5, v/v) in a non-circulating system essentially as described by Sies [17]. Bile was diluted 200-fold with water and analysed without further processing.

Samples (0.5 ml) were taken from cell suspensions in shaking culture at 37°C initially consisting of 3.5 ml of $4 \cdot 10^6$ cells per ml HBSS. Samples were carefully centrifuged for 3 min at 150 *g*, followed by separation of cells and culture medium. Cells were destroyed and proteins precipitated by one of the two following methods.

Method I. A 0.5-ml volume of 5% trichloroacetic acid (TCA) was added to the pellets. To precipitate proteins in the media, 70 μl of 40% TCA were added. All samples were placed in an ultrasonic bath for 10 min to minimize coprecipitation of BSP. Prior to analysis, samples were centrifuged (1500 *g*, 5 min). Supernatants were brought to ca. pH 2 with 20 μl of 6 *M* sodium hydroxide. The resulting solutions were submitted to analysis.

Method II. To both pellets and media, 170 μl of a mixture consisting of trichloromethane and 1-pentanol (3.5:1, v/v) were added. Furthermore, 0.5 ml of HBSS was added to the organic layer containing the destroyed cells. All samples were allowed to stand in an ultrasonic bath for 10 min and then shaken for 20 min. Samples were centrifuged and separated. The aqueous layers were analysed.

Apparatus

The ultrasonic bath used for sample preparations was a Bransonic 2200 from Branson (Soest, The Netherlands). Ultrasonic treatment of samples was performed for 30 s (amplitude 6 μm, 20 kHz) using an MSE 100-Watt ultrasonic disintegrator equipped with a titanium vibrator Microprobe, 3.2 mm, from Measuring and Scientific Equipment (London, U.K.).

HPLC assay conditions

HPLC was performed using an LKB 2150 HPLC pump as solvent-delivery system, equipped with a Rheodyne 7125 injection valve (20-μl loop) and a reversed-phase column (Chromsep C_8, 100 mm×3 mm I.D., particle size 5 μm, Chrompack, Middelburg, The Netherlands) with RP guard column.

Gradient elution was performed using a Pye Unicam LC-XP gradient programmer. The solvents used for gradient elution consisted of methanol–water: 45:55, v/v (A) and 65:35, v/v (B). Both solvents contained 2 m*M* of tetrabutylammonium hydroxide. The aqueous solution was adjusted to pH 6 with hydrochloric acid. The solvents were saturated with helium. A linear gradient was programmed from 100% A to 100% B within 6 min. Elution with 100% B was continued for 3

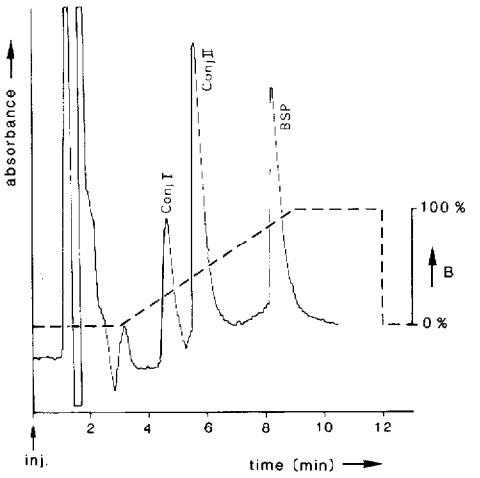

Fig. 2. Gradient profile for HPLC analysis of BSP and conjugates. The effective gradient profile, i.e. the eluent composition at the column, is shown. The 3-min delay at the gradient start is due to the volume of the tubing between the mixing chamber and the column (1.5 ml). Detection of the baseline drift shows a larger delay caused by the internal volume between the column entrance and the detector. In 6 min the eluent changes from 100% A [2 m*M* TBA in methanol–water (45:55, v/v)] to 100% B [2 m*M* TBA in methanol–water (65:35, v/v)]. The eluent was held at 100% B for 3 min, before an instant return to 100% A. The interval between two sample injections was 13 min. The retention times of BSP and of the two major metabolites are indicated.

min, followed by a final and instant return to 100% A (see gradient profile, Fig. 2). The flow-rate was 0.5 ml/min. Owing to the internal volume of the system (1.7 ml) a delay of 3.4 min between injection with gradient start and gradient detection (start of baseline drift) was observed.

A T-connection with a mixing coil was placed between the column and the detector, through which 0.05 *M* sodium hydroxide in methanol–water (60:40, v/v) was infused using an LKB 2150 HPLC pump at a flow-rate of 0.05 ml/min. Detection of BSP and BSP conjugates was achieved with an LKB 2151 variable-wavelength monitor at 578 nm.

The eluate concentrations were calculated with an LDC/Milton Roy CI-10 integrator. All analyses were performed at room temperature.

RESULTS

Three different counter-ions have been examined to separate aqueous mixtures of BSP and chemically synthesized BSP-S-G by means of ion-pair HPLC: tetramethylammonium (TMA), tetraethylammonium (TEA) and tetrabutylammonium (TBA). All three yielded acceptable retention with respect to BSP. BSP-S-G did not exhibit any retention when TMA was used, irrespective of the amount of methanol in the mobile phase.

Gradient elution reduced analysis time considerably. Compared with TBA, the use of TEA required a rather wide range in the gradient. This resulted in a comparatively large drift in baseline. Thus, we selected TBA as the pairing agent.

A gradient changing linearly within 6 min from 45:55 to 65:35 (v/v) methanol–

water, both containing 2 m*M* TBA, yielded a sufficient resolution and an analysis time of 11 min (Fig. 2). The retention times of the main peaks of conjugates and BSP were ca. 4.5, 5.5 and 9 min, respectively (Figs. 3–6).

The post-column infused alkaline solution consisted of 0.05 *M* sodium hydroxide in methanol–water (60:40, v/v) (pH ca. 12). Infusion of a methanolic solution minimized mixing problems with the mobile phase. The effluent pH was checked regularly during analysis. The use of a buffer instead of this simple alkaline solution did not improve the system.

The selectivity of the detection method could be demonstrated by interrupting the infusion of alkali. The chromatograms of samples containing BSP and conjugates then showed only solvent peaks.

A linear calibration curve ($r=0.9981$) ranging from 0.1 to 20 μM BSP in water could be constructed. Since the spectrophotometric characteristics (including molar extinction) of BSP and its conjugates are identical [1,11], sample concentrations of the dye as well as its metabolites can be related to this single curve. A sample concentration of 0.05 μM may well be quantified.

Bile samples (Fig. 3) and samples taken from cell suspensions (Figs. 4–6) were analysed using this system. The chromatograms showed peaks with resolution and retention times similar to those observed with mixtures of BSP and chemically synthesized conjugates. In addition, one or more other peaks were usually found, apparently representing other BSP conjugates. Spiking of samples (Fig. 3) showed that the retention time of the main conjugate was identical with that of the synthetic product.

Protein removal by TCA or trichloromethane–1-pentanol introduced a number of peaks partly superimposed on the solvent peak in the first 3.5 min of the chro-

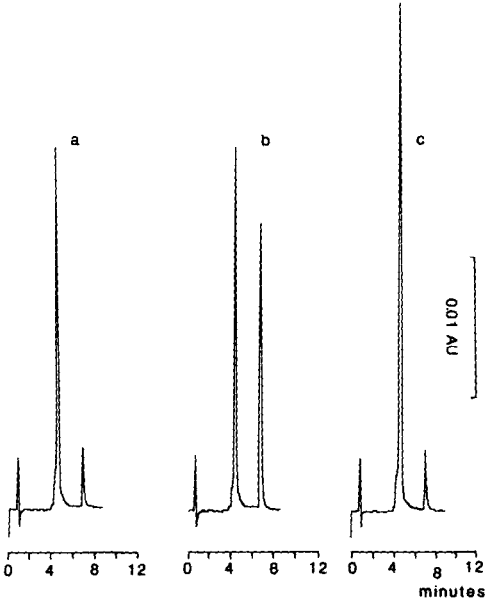

Fig. 3. HPLC analysis of BSP and conjugates in bile. Chromatograms of rat bile, diluted 200-fold with water (a.u.f.s. = 0.04): (a) bile collected from rat liver perfused with 40 μM BSP for 1.5 h; (b) as (a), but spiked with BSP; (c) as (a), but spiked with synthetic BSP-glutathione.

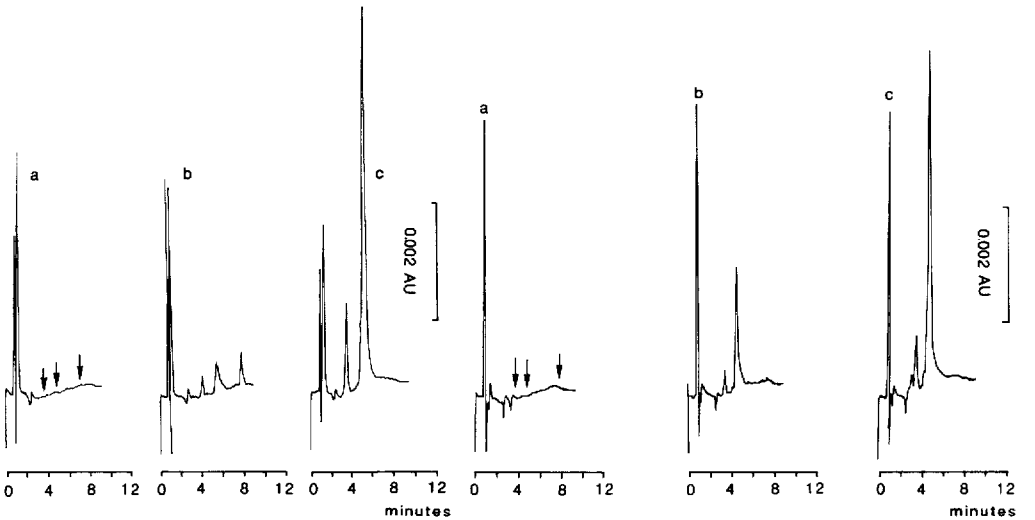

Fig. 4. HPLC analysis of BSP and conjugates in samples taken from hepatocyte cultures, deproteinized with TCA (method I). Samples were taken at 30 min from the start of incubation with 20 μM BSP. The baseline drift is the result of the gradient elution (a.u.f.s. = 0.01). (a) Hepatocyte sample to which no BSP was added; the arrows indicate positions of the main conjugates and BSP. (b) Sample of hepatocytes incubated with BSP. (c) Sample of medium after incubation of hepatocytes with BSP.

Fig. 5. HPLC analysis of BSP and conjugates in samples taken from hepatocyte cultures, deproteinized with trichloromethane-1-pentanol (method II). Samples were taken at 30 min from the start of incubation with 20 μM BSP (a.u.f.s. = 0.01). (a) Hepatocytes to which no BSP was added (positions of main conjugates and BSP are indicated by arrows). (b) Hepatocytes after incubation with BSP. (c) Medium after incubation of hepatocytes with BSP.

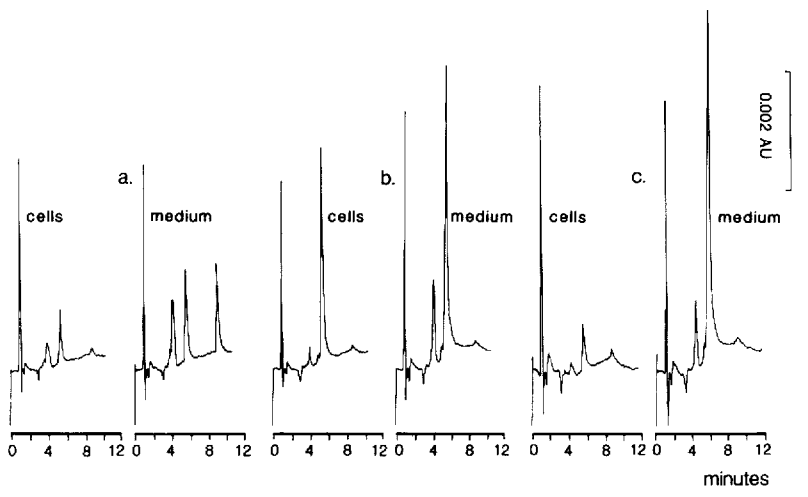

Fig. 6. Chromatograms of hepatocytes and corresponding media. Samples were taken from a cell suspension incubated with 20 μM BSP at 0, 15 and 60 min, respectively. Separation of hepatocytes and medium with subsequent deproteinization using method II (end of enzymic activity) took 4 min; the resulting effective incubation times were (a) 4 min, (b) 19 min, (c) 64 min (a.u.f.s. = 0.01).

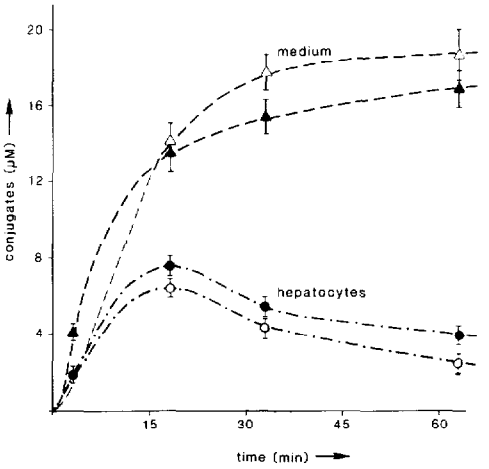

Fig. 7. Time course of concentration of BSP conjugates in hepatocytes and corresponding media. At zero time, incubation with 20 μM BSP was started. Data represent means ± S.D. of three hepatocyte culture samples from two rats: (○,△) rat 1; (●,▲) rat 2. Sample preparation method II was used. Within 20 min all BSP had been conjugated, resulting in ca. 20 μM conjugates.

matogram (Figs. 4a and 5a, respectively). Thus, a retention time of at least 3.5 min for BSP-S-G was required.

In TCA-treated samples taken from cell suspensions, only traces of BSP were detected, even with samples in which no appreciable BSP conjugation was measured (Fig. 6a). BSP recovery from pure medium (no hepatocytes added) was more than 98%. Recovery tests from solutions of 0.5 mg of BSA per ml of HBSS, after TCA treatment, yielded good results with respect to conjugates (more than 95%) but BSP recovery was poor (ca. 25%). The removal of proteins using method II yielded good recovery (more than 95%) of both BSP and conjugates from the BSA test solution. For this reason method II was the clean-up method of choice. However, a surprisingly low concentration of BSP (ca. 30% of the expected amount) was detected in cell samples. Another pool of BSP seemed to be present in cells, possibly related to the conjugation process. This pool was not accessible for analysis, either by means of deproteinization or by treatment with an ultrasonic probe, but it released BSP in the form of conjugates when sufficient incubation time was allowed (Fig. 7).

DISCUSSION

For BSP and its conjugates to be determined at 578 nm, an alkaline mobile phase is needed. The packing material of a common reversed-phase HPLC column consists of alkylated silanolic chains, which does not allow pH values over 8. Post-column alkalinization, preceding detection, was achieved by infusion of an alkaline solution. The final pH should be at least 10.8; under this condition the total amount of BSP as well as that of its conjugates can be considered coloured. Using this system no peaks were observed in any biological sample to which no BSP was added. Therefore, all peaks observed were considered to be related to BSP.

In sample chromatograms a peak for BSP and one corresponding to BSP–glutathione are observed, as can be demonstrated in samples spiked with standards of BSP and synthetic BSP-S-G, respectively (Fig. 3). In addition one or more other peaks are present near the conjugate peak of most chromatograms. Other investigators showed the existence of breakdown products or isomers of BSP–glutathione [1,4,10,11,13]. Thus, referring to the conjugates of BSP as BSP-S-G, which implies the existence of only one conjugate, might lead to misunderstanding. Combes and Stakelum [4] investigated the products of BSP conjugation in rat bile. Besides BSP–glutathione, they found its breakdown products BSP–cysteinylglycine and BSP–cysteine. Furthermore, they suggested the presence of isomers of BSP–glutathione. Quantitatively BSP–glutathione was the major metabolite. These findings were confirmed by Whelan et al. [1], who reported inter-species and inter-individual differences of BSP conjugation in rats and in guinea pigs. In humans, two metabolites with identical absorption spectra were isolated by Grodsky et al. [11], which proved to be BSP–glutathione and BSP–cysteine, possibly among others. Apparently, observed differences in metabolite patterns have to be attributed to inter-individual differences. We did not differentiate between conjugates formed in the test system, while all metabolite formation starts with glutathione coupling. Interference of the conjugation mechanism may be investigated irrespective of the final products.

Using the method described above, the BSP conjugates could be quantified with high reproducibility. However, a rapid decrease in the concentration of "free" BSP was measured in samples taken from cell suspensions. Aspecific protein binding and recovery problems might have been involved, since BSP has a high affinity for albumin [6,10,18]. However, albumin binding in vivo does not prevent a very fast clearance of the dye by the liver, although clearance is increased in the case of hypoalbuminaemia. Levi et al. [18] demonstrated the presence of proteins with an extremely high affinity for BSP in hepatocytes, which would explain the high and specifically liver-related clearance. These authors [18] quantified bound BSP by measuring concentrations of free dye that remained in the medium. Other investigators measured radioactivity resulting from labelled BSP in pellets [2,6,8]. However, these methods yield no information about the type of binding. Vonk et al. [19] described a method to extract DBSP (dibromosulphophthalein, which is very similar to BSP) from hepatocytes, which they reported to result in a 100% recovery. This method appeared to be useful for spectrophotometric measurements of BSP (results not shown) but could not be used for HPLC analysis since a rather large amount of proteins remained in the solution. Binding to proteins does not impair BSP colouring [10]. This implies that the chromophore is not directly involved in protein binding. We expect that in this case the high "recovery" of BSP is due, at least in part, to detection of protein-bound BSP.

Sample clean-up using trichloromethane–1-pentanol appears to eliminate losses due to non-specific binding to proteins, such as albumin. However, only traces of free BSP were detected in culture samples. The almost instant "loss" apparently does not originate from albumin binding. BSP is rapidly bound at or within liver

cells and cannot be released by the usual methods of sample preparation. However, conjugation still proceeds to completion (Figs. 6 and 7), which implies that BSP remains accessible for coupling with glutathione. Conjugates can be quantified after sample preparation, which indicates a different (weaker) protein binding compared with BSP. Since ultrasonic treatment does not increase the amount of BSP detected in the system, it is suggested that a covalent type of binding, rather than mere adsorption, is involved. Sulphydryl groups of glutathione-S-transferases may be the binding sites, thus forming a pool of BSP that releases only conjugated products.

BSP clearance offers a useful tool to demonstrate the effect of compounds that may interfere with liver cell functioning. To reveal the corresponding mechanisms of action of impairing compounds the cellular kinetics of BSP have to be elucidated. The clinical usefulness of the BSP liver function test in humans is limited by its inability to differentiate between intrahepatic and extrahepatic impairment. Carbone et al. [20] suggested that this differentiation could be achieved by serum determination of BSP metabolites, in addition to total BSP. The HPLC method presented here could be a convenient analytical tool to study these processes in vivo as well as in vitro.

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